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Developmental regulation of calmodulin-dependent adenylate cyclase activity in an insect endocrine gland

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The insect prothoracic gland produces ecdysteroids that elicit molting and metamorphosis, and neurohormone stimulation of steroidogenesis by this gland involves both Ca^{2+} and cyclic adenosine monophosphate second messengers. Prothoracic gland adenylate cyclase exhibits a complex Ca^{2+}/calmodulin (CaM) dependence, a component of which requires an activated $G_{s\alpha}$ for expression. A developmental switch in this system has been identified that correlates with a change in both regulation and function of the gland and involves the loss of sensitivity to extracellular Ca^{2+} at a time approximately concurrent with the loss of Ca^{2+}/CaM sensitivity by the adenylate cyclase. The extent of cholera toxin activation of gland $G_{s\alpha}$ is lowered before this developmental switch. However, no alterations in $G_{s\alpha}$ levels or mobility are detected, suggesting that $G_{s\alpha}$ interaction with another component in the signaling pathway, perhaps adenylate cyclase itself, produces the apparent Ca^{2+}/CaM dependence and influences the ability of toxin to modify $G_{s\alpha}$.

Introduction

The final larval instar of holometabolous insects is a time of massive developmental change; larval tissues are reprogrammed for the impending metamorphosis to the pupal stage, and the animal undergoes characteristic behaviors that ensure successful pupation. The prothoracic glands are critical to this process because they produce ecdysteroids, steroid hormones that are responsible for initiating both cellular and behavioral changes associated with molting and metamorphosis. In the lepidopteran *Manduca sexta,* the final (fifth) instar lasts 9 d and exhibits two hemolymph ecdysteroid peaks. On day 4 a small rise in ecdysteroid levels, termed the commitment peak, elicits wandering behavior, cessation of feeding, and reprogramming of some tissues for pupation. An ecdysteroid surge on days 7–9 initiates the process of pupal molting, pupation itself occurring on day 10.

Prothoracic gland steroidogenesis is controlled by the prothoracicotropic hormones (PTTHs), brain neuropeptides that use cyclic adenosine monophosphate (cAMP) as a second messenger (Vedeckis *et al.,* 1976; Gilbert *et al.,* 1981; Smith *et al.,* 1984). Several past observations suggest that both hormonal regulation and responsiveness of the prothoracic glands are developmentally controlled. Two forms of PTTH, distinguished by molecular weight and termed big and small PTTH, are present in the *Manduca* brain (see Gilbert *et al.,* 1981). Gland responsiveness to small PTTH changes during development, with glands from pupal insects being relatively refractory to stimulation by this form (Bollenbacher *et al.,* 1984).

Transduction of the PTTH signal is dependent on extracellular Ca^{2+} and can be mimicked by a Ca^{2+} ionophore. Therefore, it has been proposed that an influx of Ca^{2+} regulates adenylate cyclase activity, which in turn elicits an increase in the rate of ecdysteroid biosynthesis (Smith *et al.,* 1985). This hypothesis is supported by the finding of a Ca^{2+}/calmodulin (CaM)-dependent adenylate cyclase in the prothoracic gland (Meller *et al.,* 1988). One intriguing aspect of this adenylate cyclase is that Ca^{2+}/CaM regulation appears to be both complex and dependent on the animal's developmental stage. Although gland adenylate cyclase from both day 3 larvae and day 0 pupae is sensitive to Ca^{2+}/CaM, only the former glands exhibit a component of Ca^{2+}/CaM stimulation that is dependent on G protein activation (Meller *et al.,* 1988). This suggests that Ca^{2+}/CaM can interact with stimulatory guanine nucleotide–binding proteins (G_s proteins) or influence their coupling to adenylate cyclase in larval day 3 glands but not in pupal glands. The changes in Ca^{2+}/CaM sensitivity between larval and pupal stages may underlie observed differences in gland response to hor-

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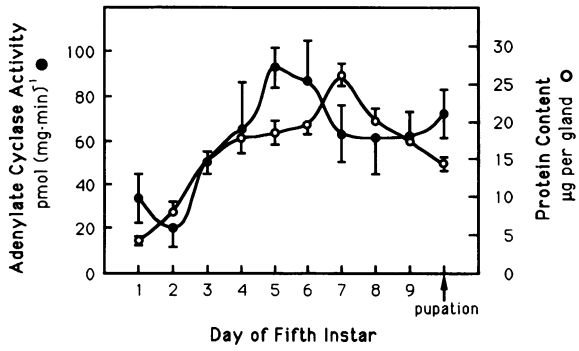

***Figure 1.* Particulate protein content and basal adenylate cyclase activity of particulate fractions of larval prothoracic glands during the final instar.** Particulate fractions were prepared as described in Materials and methods and were assayed for protein (○) or basal adenylate cyclase activity (●). Data are averages of independent means ± SE.

mones at these two stages and indicate a switch in signaling mechanisms. Modulation of the prothoracic glands signal transduction pathways would therefore determine gland function and thus be a critical step in molting and metamorphosis. In this paper both the developmental manifestation of Ca^{2+}/CaM sensitivity and its biochemical basis are explored.

Results

Gland size and enzyme activity

The prothoracic gland is composed of a homogeneous population of ~220 cells. However, because the gland changes in size during the final larval instar, we determined both the protein content of particulate preparations from these glands and the specific basal activity of adenylate cyclase in these preparations. Figure 1 shows the protein content of particulate preparations from glands throughout the instar, as well as the adenylate cyclase activity of these preparations. Glands taken on day 1 contained ~4 µg particulate protein per pair, but this value increased steadily to a maximum of >25 µg particulate protein per pair on day 7 and fell thereafter to ~14 µg by the day of pupation.

The specific activity of adenylate cyclase from these preparations underwent similar fluctuations throughout the instar. On days 1 and 2 the gland preparations produced 20–30 pmol cAMP $(mg \cdot min)^{-1}$, but this value increased to a maximum of 90 pmol $(mg \cdot min)^{-1}$ on days 5 and 6, and then decreased to ~60–70 pmol $(mg \cdot min)^{-1}$ by days 8 and 9. Because of the fluctuations in gland size and basal enzyme activity, activation ratios were used to express the results of adenylate cyclase experiments when comparisons between different developmental stages were made.

Effects of Ca^{2+} on ecdysteroid production in vitro

Calcium is required for the action of the neuropeptide PTTH on the prothoracic glands, but Ca^{2+} also affects ecdysteroid production by the glands in the absence of PTTH, as shown in Figure 2. The activation ratios for ecdysteroid production by glands incubated in media ± exogenous Ca^{2+} (6.8 mM) were determined for gland pairs taken from animals throughout the last instar. Glands from day 1–4 larvae were stimulated ~3-fold by Ca^{2+}, whereas day 5 larval glands exhibited an 11-fold stimulation. By day 7, Ca^{2+} stimulated ecdysteroid synthesis by <2-fold, and the capacity for Ca^{2+} stimulation remained low until pupation. Based on the results of this assay, the presence of exogenous Ca^{2+} alone clearly can cause an increase in ecdysteroid production.

Adenylate cyclase activation by Ca^{2+}/CaM and activated G_s protein

CaM (1 µM) may activate adenylate cyclase from day 3 glands by two discreet mechanisms: a presumed direct activation of the catalytic subunit (seen with Ca^{2+}/CaM alone), and a potentiation of G-protein–mediated stimulation (observed with Ca^{2+}/CaM in the presence of NaF or nonhydrolyzable guanosine triphosphate

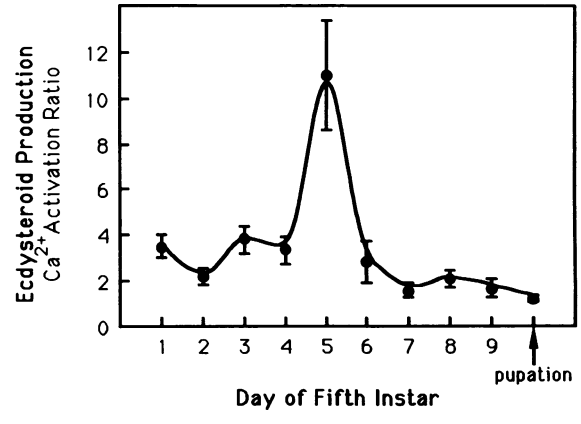

***Figure 2.* Effect of extracellular Ca^{2+} on ecdysteroid production by larval prothoracic glands during the last larval instar.** Glands were incubated with and without 6.8 mM Ca^{2+} as noted in Materials and methods and the synthesized ecdysteroids quantified by RIA. Data are expressed as the activation ratio for ecdysteroid production in the presence and absence of exogenous Ca^{2+}, experimental:basal ± SE.

[GTP] analogue) as described previously (Meller *et al.*, 1988). Figure 3 documents the developmentally regulated ability of both 1 μM CaM and G protein activators to stimulate gland adenylate cyclase. In panel A, gland adenylate cyclase is activated by 1 μM CaM (100 μM Ca^{2+} is included in all assays using CaM). Early in the instar, Ca^{2+}/CaM can cause a two- to threefold activation of adenylate cyclase, but there is a gradual onset of refractoriness to Ca^{2+}/CaM between days 4 and 7.

Figure 3B shows the response of gland adenylate cyclase to the G_s protein activators NaF and cholera toxin (CT). Activation by NaF is sensitive to developmental stage, causing an eightfold activation of adenylate cyclase from day 1 glands, but only a fourfold activation when day 3 glands are used. A similar trend with lower activation ratios is observed for CT activation.

The ability of Ca^{2+}/CaM to potentiate stimulation of adenylate cyclase by activated G_s proteins was determined throughout the final instar (Figure 3C). The solid line represents activation of adenylate cyclase by 1 μM CaM and 5 mM NaF together, and the dotted line represents the value expected if activation by these two agents was merely additive, as determined by summing the values obtained by stimulating with Ca^{2+}/CaM or NaF separately (shown in panels A and B). CaM can clearly potentiate the effect of NaF during the first half of the instar; however, this ability is lost abruptly between days 5 and 6. This is a time between the two peaks of ecdysteroid production (Bollenbacher *et al.*, 1981) when the gland is presumed to be inactive, suggesting that there is a switch in signal transduction during this time.

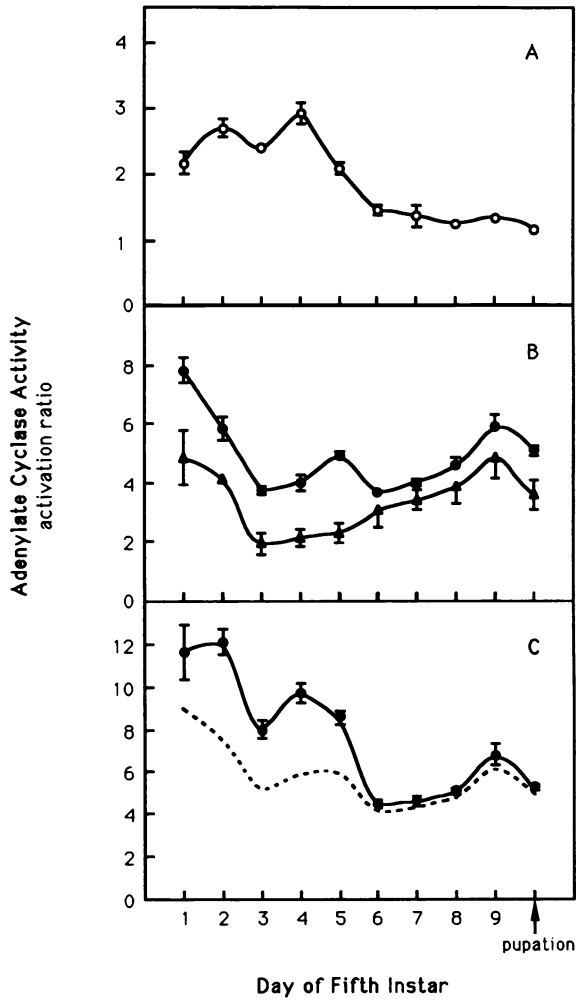

Figure 3. **Effects of Ca^{2+}/calmodulin, NaF, and CT on the adenylate cyclase activity of larval prothoracic glands during the last instar.** Prothoracic gland particulate fractions were assayed under basal conditions or in the presence of 1 μM CaM and 100 μM Ca^{2+} (A); 5 mM NaF (●) or cholera toxin preactivated (▲) (B); or with 5 mM NaF, 1 μM CaM, and 100 μM Ca^{2+} together (●) (C). The dotted line in C represents the anticipated values if NaF and Ca^{2+}/CaM activation are additive. Data are presented as the activation ratio, experimental:basal ± SE.

Relationship between NaF and CT activation

The observation that stimulation of gland adenylate cyclase by NaF and CT changes in concert prompted an analysis of the relationship between the action of these two agents. Because they have the same target, $G_{s\alpha}$, but have different modes of action, their abilities are expected to be related but not identical. A regression performed on the data from Figure 3B showed the resultant points segregating into two sets, which represent the first and second halves of the instar, shown as separate regression lines in Figure 4. The filled circles represent days 1–5, and the open circles day 6 through pupation. The segregation of data into these two groups suggests that even though the NaF and CT responses are related throughout the instar, the relative abilities of these compounds to stimulate $G_{s\alpha}$ undergoes a change after day 5. As slopes of the lines are identical, it appears that a subpopulation of the gland's $G_{s\alpha}$ proteins are inaccessible to either NaF or CT during part of the instar.

Effects of adenylyl imidodiphosphate App(NH)p

One of the potential mediators of the ability of Ca^{2+}/CaM to influence gland adenylate cyclase response to NaF is a CaM kinase. If this kinase modified a G protein or adenylate cyclase itself,

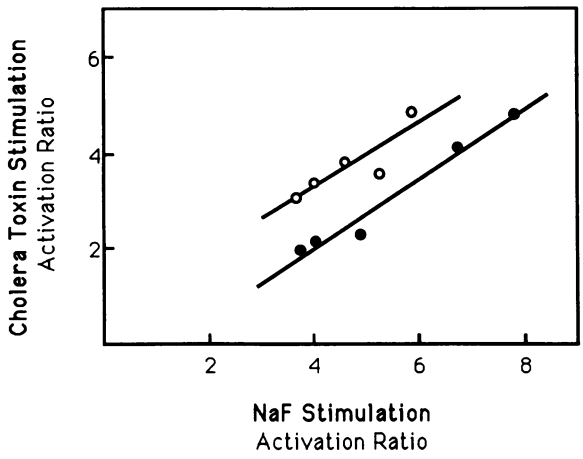

***Figure 4.* Relation between adenylate cyclase activation by NaF and CT.** For each developmental stage shown in Figure 2, the NaF and CT activation ratios are regressed. Filled symbols (●) represent days 1–5 of the last larval instar (R^2 = 0.78), and open symbols (○) represent day 6 through pupation (R^2 = 0.97; lines have significantly different elevations at the 80% confidence level).

the apparent potentiation between NaF and Ca^{2+}/CaM might be produced. To test the possibility that the Ca^{2+}/CaM supplied in our assay was activating a kinase, we performed the adenylate cyclase assay using App(NH)p as a substrate. This ATP analogue can be used by adenylate cyclase but is not a substrate for kinases, and thus would reduce or eliminate any kinase effects that are occurring because of the addition of Ca^{2+}/CaM (see Yount *et al.*, 1971). Activation ratios were determined under conditions where potentiation is seen, i.e., day 3 membranes incubated with 5 mM NaF and CaM. Table 1 demonstrates that App(NH)p has no effect on the adenylate cyclase activation ratio over a wide range of CaM concentrations, suggesting that stimulation of a kinase is not responsible for the potentiation of NaF activity by the addition of Ca^{2+}/CaM.

G protein subunits in the prothoracic gland

In an effort to determine whether patterns of G protein expression change in the prothoracic gland during the final instar, we conducted immunoblotting studies throughout this period. Figure 5 shows blots performed on glands taken from animals on day 1 through pupation, and Figure 6 reveals the relative titer of these proteins, as determined from densitometric scanning of the proteins visualized in Figure 5.

The top panel of Figure 5 depicts the results of probing with antiserum J881, which recognizes $G_{i\alpha}$ and $G_{o\alpha}$ (Mumby *et al.*, 1986). A band at 41 kDa is tentatively identified as *Manduca* $G_{o\alpha}$ by comparison with immunoblot analyses of other insect and vertebrate tissues (Meller, unpublished observations). Because all lanes in any panel of Figure 5 are loaded with the same amounts of protein, it is clear that there is no dramatic change in the titer of the 41-kDa protein throughout the instar (see also Figure 6).

The middle panel of Figure 5 shows a blot probed with antibody 83, which is specific for $G_{s\alpha}$ (Simonds *et al.*, 1989). The two proteins observed at 48 and 51 kDa in the gland particulate fraction are expressed in relatively invariant amounts throughout the instar, as determined by summing the densities of both bands (Figure 6).

The bottom panel of Figure 5 shows immunoreactivity to antiserum J99, which is specific for G_{β} (see antibody SW/1, Spiegel, 1990), and two proteins at 37 and 38 kDa are recognized. The expression of both of these proteins is apparently regulated by developmental stage, with an optimum in immunoreactivity on day 5, followed by a decline to ~40% of the maximal level in glands from newly pupated animals, as shown by the sums of the densities of these two bands in Figure 6.

Discussion

This study addresses two questions: first, what is the developmental manifestation of the changes in adenylate cyclase regulation in the prothoracic gland during the final instar? Second, what are the biochemical mechanisms underlying these observed changes? Several parameters of gland function were examined and

Table 1. Effect of App(NH)p on NaF and Ca^{2+}/CaM stimulated adenylate cyclase activity

	NaF activation ratios substrate	
CaM (μM)	ATP	App(NH)p
0	3.7 ± 0.2	3.8 ± 0.3
0.1	5.5 ± 0.7	5.1 ± 1.1
1.0	8.0 ± 0.4	7.4 ± 1.4
10	11.4 ± 1.7	11.4 ± 2.0

Particulate fractions from day 3 larval prothoracic glands were stimulated with 5 mM NaF and the indicated doses of CaM, using either 0.2 mM ATP or 0.2 mM App(NH)p as the substrate for adenylate cyclase. Calcium (100 μM) was supplied with all doses of CaM. Data are the averages of means ± SE from three separate experiments.

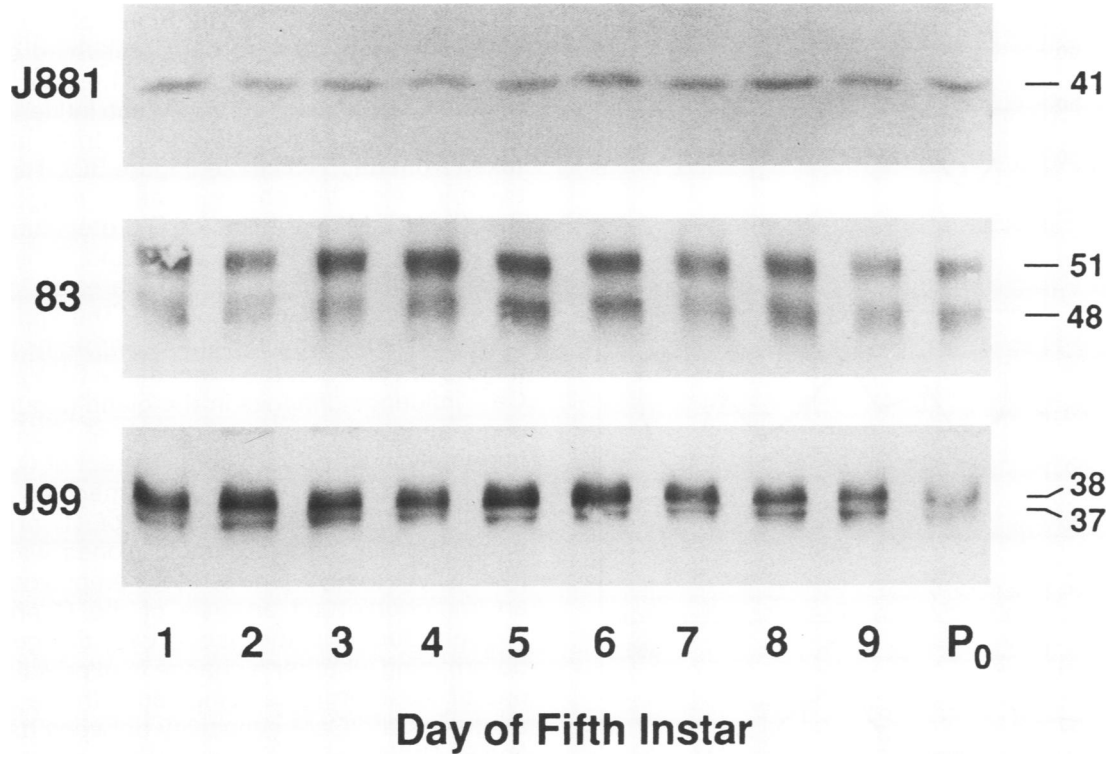

Figure 5. **G protein immunoreactivity in prothoracic glands during the fifth instar.** Western blot lanes are gland particulate preparations taken from insects on day 1 of the final instar and continuing until pupation. The top panel has 30 μg of protein per lane, and is probed with antiserum J881 (G_α specific). The middle panel has 40 μg of protein per lane, and is probed with antibody 83 ($G_{s\alpha}$ specific). The bottom panel has 30 μg of protein per lane, and is probed with antiserum J99 (G_β specific). Molecular weight $\times 10^{-3}$ is shown on the right.

were found to be quite dynamic. The ability of Ca^{2+} to stimulate ecdysteroid production by whole glands in vitro changes dramatically during the larval instar, as does Ca^{2+}/CaM sensitivity of gland adenylate cyclase. The ability of Ca^{2+}/CaM to potentiate NaF activation of adenylate cyclase is lost midway through the instar, and the relative efficacies of NaF and CT stimulation of adenylate cyclase change at the same time. These observations indicate that a fundamental change in control of adenylate cyclase may underlie developmental regulation of the prothoracic gland. To the best of our knowledge, this is the only system in which such a change can be correlated with tissue function.

Extracellular Ca^{2+} alone can increase ecdysteroid production by glands in vitro early in the instar; and the degree of stimulation peaks on day 5, followed by a rapid fall and total loss of gland sensitivity to Ca^{2+} by the time of pupation. The absence of PTTH from these incubations leads to the conclusion that there is a PTTH-independent pathway by which Ca^{2+} can stimulate the glands either directly or by removing an inherent inhibition during the first half of the final instar. Calcium, therefore, functions in two capacities to stimulate the prothoracic glands: activation by PTTH has been shown to be Ca^{2+}-dependent (Smith *et al.,* 1985), and activation during the first half of the fifth instar can be achieved with Ca^{2+} in the absence of PTTH (Figure 2). The mechanism of this second function of Ca^{2+} is still obscure. The striking effect of Ca^{2+} on day 5, when the gland is presumed to be quiescent, suggests that multiple changes in signal transduction occur in an asynchronous fashion between the two ecdysteroid peaks, e.g., an increase in Ca^{2+} permeability or release of an inhibitory pathway after day 4, followed by deletion of a pathway for Ca^{2+} stimulation after day 5. Data presented in this paper offer substantial evidence that this latter change is occurring between days 5 and 6. Previous comparisons of endogenous CaM levels in glands from day 3 animals and pupae show that the CaM titer in both is ~15 μM, even though enzyme response to added CaM is very different (Meller *et al.,* 1988). Calcium dose response

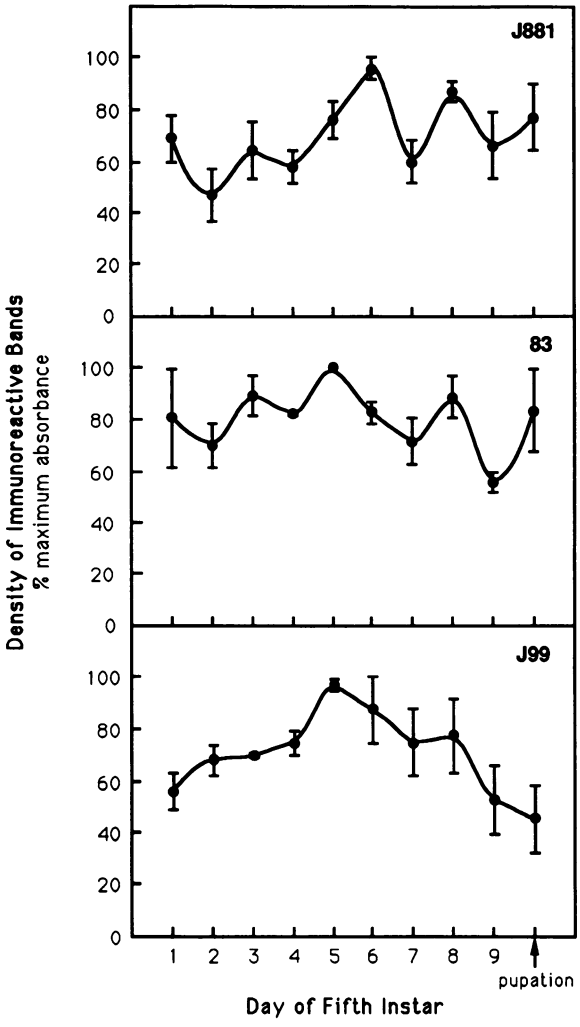

Figure 6. **Developmental expression of G proteins in the prothoracic gland.** A series of blots similar to those shown in Figure 5 were densitometrically quantified and the data normalized to maximal adsorbance = 100%. Top panel shows the means ± SE of four blots probed with antiserum J881 (G_α specific). Middle panel shows the means ± SD of two blots probed with antibody 83 ($G_{s\alpha}$ specific). Bottom panel shows the means ± SE of three blots probed with antiserum J99 (G_β specific).

data from larval and pupal animals suggest that near-optimal levels of Ca^{2+} are present in preparations from both these stages, but in both cases slight increases in activity could be achieved with the addition of 100 μM Ca^{2+} (unpublished data), suggesting that the endogenous levels of Ca^{2+}/CaM are not responsible for the developmental differences observed in response to CaM. Although we believe that a role for Ca^{2+} in the synthesis of ecdysteroids is likely, it would be stage neutral and not responsible for the dramatic changes observed in Figure 2.

Not only can Ca^{2+} act through CaM to stimulate adenylate cyclase by a presumed direct mechanism during the first half of the final instar (Figure 3A), but also Ca^{2+}/CaM can influence G_s stimulation of adenylate cyclase from glands taken from day 1–5 larvae (Figure 3C). There is evidence from vertebrate systems suggesting that Ca^{2+}/CaM may influence G protein function (Jackowski *et al.*, 1980; Gnegy and Treisman, 1981; Asano *et al.*, 1986; Katada *et al.*, 1987; Perez-Reyes and Cooper, 1987). The apparent change in sensitivity of gland $G_{s\alpha}$ proteins to CT (Figure 4) occurs at the exact same time as the Ca^{2+} influence changes in the gland (Figure 3), suggesting that the developmental change is intimately involved with some aspect of G_s protein expression or function. The mechanism of putative Ca^{2+}/CaM modulation of G_s protein stimulation in the prothoracic glands was explored in two ways. One strategy employed was an immunoblotting analysis of the G protein subunits found in the gland, with the goal of determining whether a change in expression of one of these proteins coincided with the alterations in Ca^{2+}/CaM sensitivity between days 5 and 6. The second approach assumed that the effects of Ca^{2+}/CaM supplied in our assays might be through a CaM kinase that modified a component of the signal transduction pathway. Involvement of a CaM kinase was deemed unlikely by the use of App(NH)p as a substrate for day 3 adenylate cyclase. The data from these experiments (Table 1) showed qualitatively identical responses to NaF over a wide range of CaM concentrations when either adenosine triphosphate (ATP) or App(NH)p was used as a substrate. This suggests that the exogenous Ca^{2+}/CaM is interacting directly with some component of the signal transduction system, resulting in the facilitation of G_s protein function.

Immunoblotting studies of G protein subunits in the prothoracic gland revealed two proteins of 48 and 51 kDa reacting with antibody 83; these proteins appear to be the *Manduca* form of $G_{s\alpha}$. The carboxy terminus of $G_{s\alpha}$ to which antibody 83 is raised is virtually identical between *Drosophila* and bovine brain (Quan *et al.*, 1989), allowing recognition of the insect protein. Antibody 83 can immunoprecipitate adenylate cyclase activity from detergent extracts of *Manduca* glands (Meller, unpublished data), demonstrating that the 48- and 51-kDa proteins may be functionally equivalent to $G_{s\alpha}$ also. These studies also reveal a protein of 41 kDa that reacts with antiserum J881 and is tentatively identified as *Manduca* $G_{o\alpha}$ (unpublished observations). The use of antiserum J99 revealed two

potential *Manduca* G_β subunits of 37 and 38 kDa. None of these proteins displayed qualitative differences in expression during the 10-d developmental period studied, and only G_β showed a consistent quantitative difference in expression (see Figures 5 and 6). The relatively constant titer of α subunits is surprising in light of the massive changes in both gland size and adenylate cyclase activity during the period investigated. As shown in Figure 1, the gland protein content increases fivefold between days 1 and 7, and the specific activity of adenylate cyclase in tissue preparations increases fourfold between days 1 and 5, suggesting that adenylate cyclase is being expressed at disproportionately high levels during the middle of the instar. As the insect $G_{s\alpha}$ homologue is constant in expression during this period, the ratio of adenylate cyclase to $G_{s\alpha}$ could be decreasing between days 1 and 5 and increasing thereafter. This may underlie part of the fluctuations observed in the stimulation of adenylate cyclase through the $G_{s\alpha}$ activators NaF and CT.

The presence of a putative $G_{o\alpha}$ in the prothoracic gland raises the question of the actual role of the PTTHs, as well as other possible ecdysteroidotropic signals. Evidence that big PTTH is directly coupled to adenylate cyclase remains elusive, and therefore it or another hormone may be coupled to an ion channel through this α subunit. $G_{o\alpha}$ has been implicated in both control of Ca^{2+} and K^+ channels (Gilman, 1989), either of which may play important roles in prothoracic gland regulation.

An apparent alteration in G protein function, which was not clarified by the present immunoblotting studies, was the change in relative efficacies of NaF and CT in stimulating adenylate cyclase from larval glands between days 5 and 6. CT activates $G_{s\alpha}$ proteins by the covalent attachment of the ADP-ribose derived from NAD^+ (Cassel and Pfeuffer, 1978); this enzymatic reaction is both very selective of its substrate and sensitive to nucleotides, cations, and ADP-ribosylation factors (Tsai *et al.,* 1987; Price *et al.,* 1988; Tsuchiya *et al.,* 1989). Because NaF activation (Gilman, 1984) is nonenzymatic and relatively insensitive to conditions or the G protein involved, we attribute the change in relative efficacies of NaF and CT between days 5 and 6 to an increase in the ability of CT to activate $G_{s\alpha}$. The two lines with the same slope and different elevations in Figure 4 suggest that there are two pools of $G_{s\alpha}$ in the gland before day 5 that differ in their sensitivity to CT activation. CT may discriminate against either a different, posttranslationally modified $G_{s\alpha}$ protein or against one closely associated with an effector or receptor that sterically blocks CT labeling. Using both one- and two-dimensional electrophoresis, we have been unable to detect qualitative changes in the putative $G_{s\alpha}$ expressed between the first and second halves of the last larval instar. In addition, we observed no differences in CT labeling patterns of glands from days 1 and 9 (Meller, unpublished observations), suggesting that the $G_{s\alpha}$ homologues in the prothoracic gland are invariant in expression. If a protein associated with G_s is capable of influencing CT activity, the most obvious candidate in our system is adenylate cyclase itself.

Adenylate cyclase exists in both CaM-sensitive and -insensitive forms (Westcott *et al.,* 1979; Rosenberg and Storm, 1987; Mollner and Pfeuffer, 1988), and CaM-dependent adenylate cyclase also occurs in insects (Dudai and Zvi, 1984; Livingstone *et al.,* 1984; Combest *et al.,* 1985). Indeed, a CaM-dependent adenylate cyclase gene implicated in the learning of *Drosophila* has been cloned by the use of a bovine adenylate cyclase as a probe (Krupinski *et al.,* 1989). Developmental changes observed in the *Manduca* prothoracic gland system suggest that different forms of this enzyme may be expressed as the final instar progresses. The data presented in Figure 2A indicate a loss of Ca^{2+}/CaM sensitivity by the adenylate cyclase between days 4 and 6 of the last instar. As the level of CaM used in this experiment, 1 μM, is presumed to act directly on the catalytic unit, the simplest interpretation of the data is that a Ca^{2+}/CaM-dependent form of adenylate cyclase is expressed during the first half of the instar but not after day 5. The ability of Ca^{2+}/CaM to potentiate NaF stimulation is lost at about the same time, suggesting that the same biochemical change may be responsible for both these events and may in addition underlie the observed loss of gland stimulation by exogenous Ca^{2+} at the end of the instar. It has been suggested that CaM potentiates the response of CaM-dependent brain adenylate cyclase to activated $G_{s\alpha}$ proteins (Harrison *et al.,* 1989). Using this as a model to reinterpret the complex dose response of prothoracic gland adenylate cyclase to Ca^{2+}/CaM during the first half of the instar (see Meller *et al.,* 1988), we propose that Ca^{2+}/CaM is acting at two sites on adenylate cyclase, a high-affinity site independent of G_s and a lower affinity site that is silent in the absence of activated G_s.

Although the specific roles of the different forms of PTTH are unclear, small PTTH may activate a signaling pathway that is lost after the

commitment peak, because gland sensitivity to this form decreases between day 3 and pupation (Bollenbacher *et al.,* 1984). This pathway could involve the effects of Ca^{2+}/CaM on gland adenylate cyclase that are discussed above. Although the release of PTTH is generally accepted as the principal factor controlling prothoracic gland activity (Gilbert *et al.,* 1988), there is evidence for multiple inputs regulating the precise onset of the commitment peak (Richter, 1989; Mizoguchi and Ishizaki, 1982).

The small hemolymph ecdysteroid peak on day 4 of the last larval instar is perhaps one of the most critical points in the development of *Manduca,* because it signals the cessation of larval feeding and elicits other behavioral changes and the change in commitment required for metamorphosis to the pupa (see Riddiford, 1976). If transduction of a signal from receptor to adenylate cyclase is dependent on Ca^{2+}/CaM during the ecdysteroid commitment peak, this suggests a mechanism whereby multiple neural or endocrine signals may be integrated. We observed previously that guanine nucleotide analogues were unable to stimulate adenylate cyclase from day 3 larval glands immediately prior to the commitment peak in the absence of Ca^{2+}/CaM, but were effective in stimulating the adenylate cyclase of pupal glands. This lead to the hypothesis that a Ca^{2+} mobilizing signal could exert a permissive or enhancing effect on the transduction of signals to receptor coupled adenylate cyclase early in the fifth instar (Meller *et al.,* 1988). The present data would suggest that this elaborate regulatory system is dismantled between the ecdysteroid commitment and prepupal peaks.

In summary, we have identified a dynamic change in a unique, Ca^{2+}/CaM-dependent signal transduction pathway in a steroid-producing endocrine gland. This change occurs at a developmentally important time, midway between two ecdysteroid peaks that have very different morphogenic functions. This has implications for both the physiology of ecdysteroid production by the insect prothoracic gland and the biochemistry of signal transduction in general.

Materials and methods

Animals

Manduca sexta larvae were reared individually on artificial diet at 26°C in high humidity (>60%), under a nondiapausing light regimen (light:dark ratio 16:8 h) (Vince and Gilbert, 1977). Insects were staged by a combination of developmental markers, timing, and weight. Day 1 animals were those that molted to the fifth instar between 5 and 7 PM the previous day. For days 2–4, animals conformed to the following weight classes: day 2 animals were between 2 and 4 g, day 3 between 5 and 7 g, and day 4 between 8 and 10 grams. Day 5 animals were chosen by observing the exposed dorsal vessel and pink coloration on the dorsal mid-line, and days 6–8 larvae were staged on day 5. Day 9 animals had lightly sclerotized thoracic bars (Goodman *et al.,* 1985), and day 0 pupae (P_0) were taken <24 h after pupation. All dissections were performed before 1 PM on the days indicated.

Tissue preparation

For the adenylate cyclase assay, prothoracic glands were extirpated under lepidopteran saline (Weevers, 1966), frozen on dry ice immediately, and stored at −85°C until use. To prepare membrane fractions, we homogenized groups of 100 glands in an all-glass homogenizer with 200 μl of buffer consisting of 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, pH 7.2), 0.25 M sucrose, 1 mM EDTA, 5 mM $MgCl_2$, 20 mM benzamidine, and 100 μM leupeptin. The homogenizer was rinsed with another 200 μl of the above buffer; the combined homogenate was centrifuged 15 min at 30 000 × *g;* and the pellet resuspended in a total of 400 μl buffer containing 10 mM HEPES (pH 7.2), 1 mM EDTA, 1 mM 1,4-dithiothreitol (DTT), 20 mM benzamidine, and 100 μM leupeptin. This was centrifuged again at 30 000 × *g* for 15 min; and the pellet was resuspended to a protein concentration of 0.7–1.6 mg/ml in buffer containing 10 mM sodium-potassium phosphate (pH 7.2), 1 mM DTT, 1 mM EDTA, and 100 μM leupeptin. Aliquots of this preparation (10 μl, 7–16 μg protein) were used to initiate the adenylate cyclase assay.

Reagents

CT (whole toxin) was purchased from Calbiochem (La Jolla, CA) and Grace's Ca^{2+}-free insect medium was obtained from GIBCO (Grand Island, NY). The ATP analogue App(NH)p, nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indoylphosphate (BCIP), and goat anti-rabbit alkaline phosphatase conjugated secondary antibody were purchased from Sigma (St. Louis, MO). All reagents used in the adenylate cyclase assay were as previously described (Meller *et al.,* 1988).

Adenylate cyclase assay

The assay modified from Combest *et al.* (1985) measures the conversion of [^{32}P]ATP to [^{32}P]cAMP, and includes an ATP-regenerating system in the reaction mixture. The reaction proceeded for 30 min at 30°C in a total volume of 100 μl. The reaction mixture contained 50 mM HEPES (pH 7.2) 6 mM $MgCl_2$, 0.5 mM 1-methyl-3-isobutylxanthine (MIX), 0.1% bovine serum albumin (BSA; w/v), 2 mM creatine phosphate, 0.8 mM EDTA, 10 μg myokinase, 10 μg creatine kinase, 0.40 μg adenosine deaminase, 0.2 mM ATP, and 1.0 μCi of α[^{32}P]ATP. Column separation of cAMP and ATP was performed as described (Combest *et al.,* 1985), and recoveries were typically 70%.

CT activation of adenylate cyclase

The CT activation ratio was calculated from the adenylate cyclase activity of membrane fractions pretreated with CT divided by that from control membrane fractions that were incubated under identical conditions with carrier buffer substituting for the CT (200 mM Tris [pH 7.5], 200 mM NaCl, 1 mM EDTA, and 3 mM sodium azide).

CT was preactivated in 50 mM DTT at 30°C for 15 min, and a 10-μl aliquot containing 5 μg of toxin was used to initiate adenosine diphosphate (ADP)-ribosylation. The reaction mixture contained 50 mM tris(hydroxy-

methyl)aminomethane (Tris) buffer (pH 7.2), 50 μM GTP, 200 μM ATP, 1 mM NAD^+, 0.8 mM EDTA, 2 mM $MgCl_2$, 0.1% BSA, and 200 μM leupeptin. It also contained a nucleotide-regenerating system consisting of 100 μg/ml creatine kinase, 100 μg/ml myokinase, and 2 mM creatine phosphate. Activation was achieved in a 50-μl volume containing washed particulate fractions at a concentration of 3–6 mg/ml. The reaction was allowed to continue for 30 min at 30°C and was terminated by adding 500 μl ice-cold 100 mM Tris (pH 7.2). Treated tissue was recovered by centrifugation at 30 000 × *g* for 15 min, and the resultant pellet suspended in 100 μl of buffer containing 10 mM sodium-potassium phosphate (pH 7.2), 1 mM DTT, 1 mM EDTA, and 100 μM leupeptin. Aliquots (10 μl) were then used to initiate the adenylate cyclase assay.

Prothoracic gland incubation and ecdysteroid radioimmunoassay

Ecdysteroid production was determined by dissecting glands under Ca^{2+}-free Grace's medium, and incubating individual glands in 25-μl droplets of Ca^{2+}-free Grace's or Grace's supplemented with 6.8 mM $CaCl_2$. After 2 h, aliquots of the medium were removed and ecdysteroids measured by radioimmunoassay (Warren and Gilbert, 1988).

Antibodies and antisera

Antiserum J881 (raised to a conserved G_α sequence) was the gift of Drs. S. Mumby and A. Gilman (University of Texas Medical Center, Dallas, TX) (see Mumby *et al.*, 1986), and it was used at a dilution of 1:500. Antibody 83 (an affinity-purified $G_{s\alpha}$ carboxy terminal specific antibody; see antibody RM, Simonds *et al.*, 1989), and antiserum J99 (G_β carboxy-terminal specific; see antibody SW/1, Spiegel, 1990) were the gift of Dr. Alan Spiegel (National Institutes of Health, Bethesda, MD). Antibody 83 was used at a concentration of 10 μg/ml and J99 at a dilution of 1:400. All antibodies were raised in rabbits with synthetic peptides that represent epitopes of the mammalian protein.

Immunoblotting techniques

Sodium dodecyl sulfate gels were blotted onto nitrocellulose, rinsed briefly in Tris-buffered saline with 0.2% Tween-20 (TBST), and blocked in this buffer with 0.5% milk solids and 2.5 mM sodium azide (blocking buffer) for 20 min. Blots were then incubated in the appropriate primary antiserum or antibody diluted in blocking buffer, either overnight (for 83 and J99) or for 1–2 h (for J881). Blots were rinsed four times for 5 min each with TBST, and incubated 1 h in goat anti-rabbit alkaline phosphatase conjugated secondary antibody (Sigma) at a 1:1000 dilution in blocking buffer. Blots were washed as before, rinsed in distilled water, and developed in an NBT and BCIP color reagent.

Protein determinations

The protein content of tissue fractions was determined as described by Lowry *et al.* (1951).

Data presentation and statistics

Activation ratios are calculated by dividing the value obtained when a specified treatment was used by the basal value. All data points are means ± SE of between 3 and 12 independent determinations. The regression lines and R^2 values presented in Figure 4 were computer generated, and the test used to determine difference in elevation of the lines shown is that described by Zar (1984). Because data in the two regressed samples were unpaired, mean values were used, yielding a sample size of 10, and a two-tailed *t* test was performed.

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